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Degradation of FAT10 by the 26S proteasome is independent of ubiquitylation but relies on NUB1L

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The ubiquitin-like modifier FAT10 targets proteins for degradation by the proteasome, a process accelerated by the UBL-UBA domain protein NEDD8 ultimate buster 1-long. Here, we show that FAT10-mediated degradation occurs independently of poly-ubiquitylation as purified 26S proteasome readily degraded FAT10-dihydrofolate reductase (DHFR) but not ubiquitin-DHFR in vitro. Interestingly, the 26S proteasome could only degrade FAT10-DHFR when NUB1L was present. Knock-down of NUB1L attenuated the degradation of FAT10-DHFR in intact cells suggesting that NUB1L determines the degradation rate of FAT10-linked proteins. In conclusion, our data establish FAT10 as a ubiquitin-independent but NUB1L-dependent targeting signal for proteasomal degradation.

Keywords:
Ubiquitin
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1. Introduction

The 26S proteasome is responsible for the turnover of most proteins in eukaryotes. Proteins are targeted for proteasomal degradation by the covalent attachment of a chain of ubiquitin molecules. Substrate recognition by the proteasome is mediated by five subunits of the 19S regulator: S5a/Rpn10, S6′/Rpt5, and Rpn13/ARM1 which directly recruit K48-linked polyubiquitin chains as well as S1/Rpn2 and S2/Rpn1, which indirectly recognize substrates by binding to linker proteins such as Rad23/hHR23 and Dsk2/hPLIC [1]. Besides this canonical mode of proteasomal degradation, a few alternative routes into the proteasome exist. The most prominent case is that of ornithine decarboxylase (ODC), the rate-limiting enzyme in the synthesis of polyamines. Cellular ODC levels are controlled through a negative feedback mechanism involving the polyamine-induced inhibitor antizyme (AZ) [2].

A different case is that of the ubiquitin-like modifier FAT10, which encompasses two ubiquitin-like domains. FAT10 lies encoded in the major histocompatibility complex class I locus and is inducible with IFN-γ and TNF-α [3,4]. FAT10 is activated by the E1 enzyme UBA6 (E1-L2, UBE1L2) and becomes conjugated to so far unidentified target proteins through a free diglycine-motif at its C-terminus [5,6]. N-terminal fusions of FAT10 to long-lived proteins such as GFP and dihydrofolate reductase (DHFR) are degraded with a kinetic similar to that of ubiquitin-fusions while SUMO-1 fusions remain stable suggesting that FAT10 serves as a signal for proteasomal degradation [7,8]. Degradation of FAT10 and FAT10-linked proteins can be further accelerated by the UBL-UBA domain protein NEDD8 ultimate buster 1-long (NUB1L), which interacts with both FAT10 and the 26S proteasome [8].

A pertinent question is whether FAT10 can mediate proteasomal degradation independently of the ubiquitin system. Two approaches have been used to address this issue in intact cells: the first was the mutation of all 17 lysine residues to arginine which did not affect proteasomal degradation of FAT10 [8]. Although we could not detect any ubiquitylation of lysineless FAT10, one can not exclude that residual ubiquitylation of the N-terminus of FAT10 below detection level may contribute to proteasome targeting. The second approach was the investigation of FAT10 degradation in E36ts20 cells, which bear a temperature sensitive mutation of the ubiquitin activating enzyme UBE1. In these cells FAT10 degradation was normal at the restrictive temperature [8]. However, the recent discovery of UBA6 as a second ubiquitin activating enzyme [9,10,6] opens the possibility that ubiquitylation of FAT10 relies on UBA6 thus rendering experiments in ts20 cells inconclusive.

Here, we have addressed whether FAT10 can target a protein to the 26S proteasome independently of ubiquitylation in vitro. We found that an N-terminal FAT10-DHFR fusion protein can be

Abbreviations: AZ, antizyme; DHFR, dihydrofolate reductase; NUB1L, NEDD8 ultimate buster 1-long; ODC, ornithine decarboxylase

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degraded in vitro by purified 26S proteasomes, but only in the presence of NUB1L. The herein presented results conclusively show the ubiquitin-independence of FAT10-mediated degradation and, given that FAT10 can also directly interact with the proteasome [11], allow the conclusion that mere association of FAT10 with the 26S proteasome is not enough to promote its degradation.

2. Materials and methods

2.1. Proteasome purification

Purification of the 20S proteasome was performed as described elsewhere [11], the 26S proteasome was purified as previously described [12] with modifications as outlined in the Supplementary material.

2.2. In vitro degradation assays

Protein degradation assays were performed as previously outlined [12] with some modifications. The assays were performed in 50-µl reaction volumes at 37 °C for one hour and contained 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM ATP, 20% glycerol, an ATP regenerating system (2 mM dithiothreitol, 10 mM creatine phosphate, 1.6 mg/ml creatine kinase), 1 mg/ml acetylated bovine serum albumin (Promega) and proteasomes. Reactions were initiated by addition of substrate (usually 20000 cpm) and quenched by addition of 50 µl of 20% (w/v) trichloroacetic acid. The insoluble material was removed by centrifugation (14000×*g*, 15 min), and 3 × 30 µl of the supernatant were measured in a scintillation counter. Percentage degradation of radiolabeled proteins was determined as the released counts/min divided by total input in counts/min. Background counts/min were determined in reactions devoid of proteasomes, and were typically 1–3% of total input counts.

2.3. Western blot and immunoprecipitation

Determination of NUB1L expression was performed by western blotting using anti-NUB1L peptide antibodies provided by Biomol and Dr. Michal E. Cheetham (London) [13]. Cells were harvested, washed, and lysed in 200 µl 50 mM Tris–HCl, pH 7.8, 0.01% SDS. After sonication and centrifugation, the supernatant was removed and the OD at 280 nm determined. Lysates were diluted to an OD_{280} of 0.15 and 10, 5 and 1 µl was loaded onto SDS–PAGE. As a loading control the HSP90-specific antibody H-114 (Santa Cruz Biotechnology) was used. Cultivation and transfection of HeLa-cells, pulse-chase analysis, immunoprecipitation, SDS–PAGE and audioradiography have been described before [7].

3. Results and discussion

3.1. Degradation of FAT10-DHFR in vitro is independent of ubiquitylation but relies on NUB1L

In order to investigate whether purified 26S proteasome can degrade a FAT10-linked protein in the absence of ubiquitylation we used an N-terminal fusion of FAT10 to DHFR as a model substrate, because Ub_5-DHFR can be degraded by the 26S proteasome in vitro [14] and because an HA-FAT10-DHFR fusion protein was degraded in a proteasome-dependent manner in intact cells [8]. To exclude any residual ubiquitylating activity in our reactions, we decided on Ub-DHFR as a negative control. Ub-DHFR is readily polyubiquitylated and degraded by the proteasome [8,15], however, a single ubiquitin moiety is insufficient to promote degradation by 26S proteasomes in vitro [14]. A C-terminal fusion of FAT10 to GST (GST-FAT10) was also included as a test substrate because the C-terminal attachment of K48R mutated ubiquitin to DHFR left this substrate relatively stable compared to the degradation of an N-end rule substrate [16].

FAT10-DHFR, Ub-DHFR, and GST-FAT10 were produced in *Escherchia coli* and metabolically labeled with [^{35}S]-methionine. Degradation was measured by the release of acid soluble counts and was linear over time (data not shown). [^{35}S]-labeled, in vitro transcribed/translated and affinity-purified ODC served as the positive control for degradation. The proteinaceous components of our in vitro degradation assays are depicted in Fig. 1. In panel A a silver staining of the purified proteins is shown while in panel B the autoradiogram of the same proteins is presented.

Degradation of ODC already occured to a minor extent when incubated with 26S proteasome alone (Fig. 2A, compare bars 1 and 3) and could be increased by the addition of AZ (Fig. 2A, bar 4). Incubation with 5 µM of the proteasome inhibitor epoxomicin reduced degradation to background levels (Fig. 2A, bar 5). Fig. 2B reveals our assay to be devoid of ubiquitylating activity, as Ub-DHFR was not degraded by the proteasome under any of the conditions investigated.

Previously, we showed that FAT10 directly binds the 26S proteasome [11], however, incubation of FAT10-DHFR with 26S proteasome alone was insufficient to promote its degradation (Fig. 2C, bar 2) which was as low as in the presence of epoxomicin (Fig. 2C, bar 5). Remarkably, the addition of purified, recombinant NUB1L induced degradation of FAT10-DHFR by the 26S proteasome in a dose-dependent manner (Fig. 2C, bars 3 and 4). In contrast, FAT10-DHFR was completely stable when incubated with purified 20S proteasome (Fig. 2C, bars 8 and 9). Experiments with GST-FAT10 showed that C-terminal fusion of FAT10 was insufficient to promote degradation of GST despite the ability of GST-FAT10 to interact with both NUB1L and the 26S proteasome in vitro ([7,11] and data not shown).

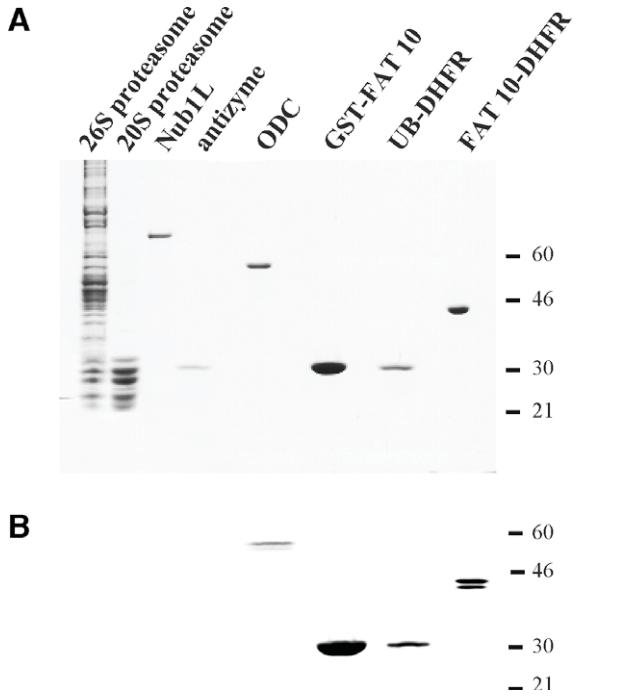

Fig. 1. Purified components for in vitro degradation. (A) Silver stained SDS–gel of purified 26S proteasome (about 500 ng), purified 20S proteasome (about 250 ng) and the recombinant proteins NUB1L, antizyme, ornithine decarboxylase (ODC), GST-FAT10, ubiquitin-dihydrofolate reductase (Ub-DHFR) and FAT10–DHFR (between 10 and 50 ng each). (B) Autoradiogram of radioactively labelled ODC, GST-FAT10, Ub-DHFR, and FAT10-DHFR.

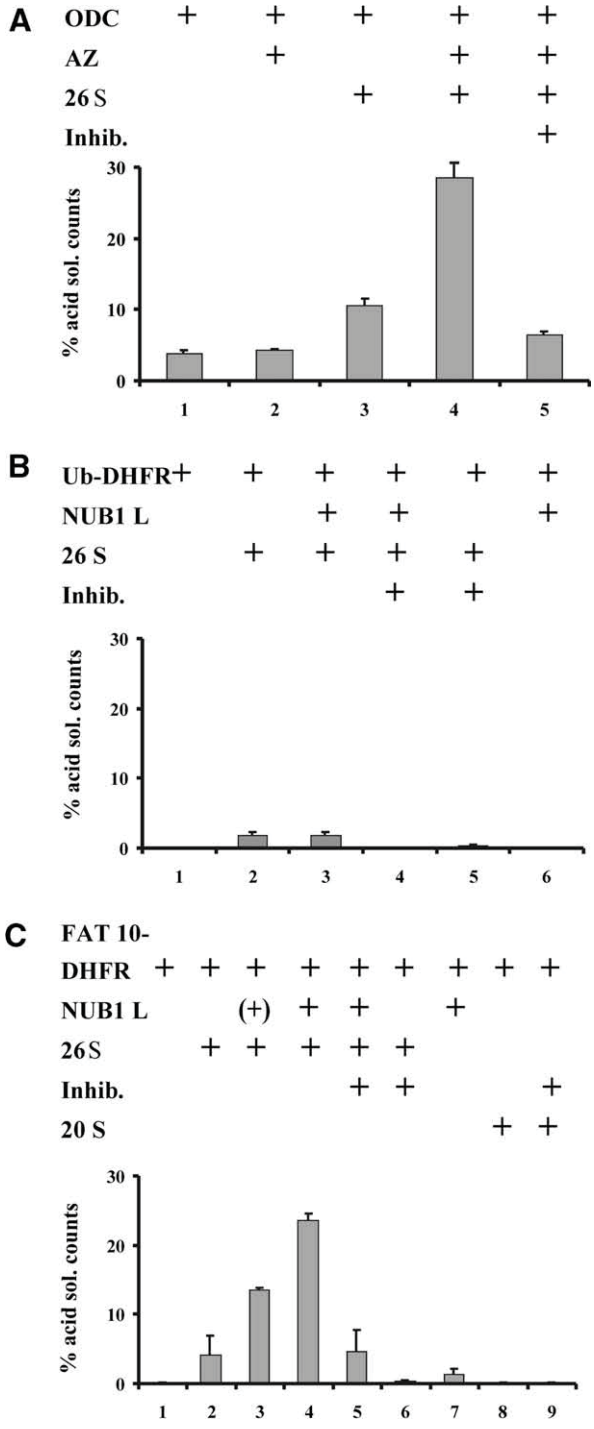

Fig. 2. Degradation of FAT10-DHFR by the 26S proteasome in vitro is independent of ubiquitylation but relies on NUB1L. The *Y*-axis always represents percent of acid soluble counts released per hour. The in vitro reactions were performed with the components indicated above each panel. Inhib. refers to degradation occurring in the presence of 5 μM of the specific proteasome inhibitor epoxomicin. (A) Antizyme (AZ)-dependent degradation of ornithine decarboxylase (ODC) as positive control for 26S proteasome activity. (B) Ubiquitin-DHFR (Ub-DHFR) is not significantly degraded by the purified 26S proteasome. (C) NUB1L-dependent degradation of FAT10-DHFR by purified 26S proteasome. A plus sign in brackets (+) and a non-bracketed + sign designate degradation in the presence of low (2 ng) and high (20 ng) amounts of NUB1L, respectively.

3.2. NUB1L is required for FAT10-DHFR degradation in vivo

To determine whether NUB1L is essential for the degradation of FAT10-DHFR in vivo, we generated HeLa cell transfectants stably expressing two shRNAs directed against NUB1L. Based on quantitative RT-PCR analysis, we selected three clones for pulse-chase experiments with knock-down efficiencies of 91.2% (clone G6), 90.5% (clone F9) and 89.1% (clone H10), respectively. Western blots of cell lysates probed with two different antibodies against NUB1 as well as an anti-HSP90 antibody as loading control revealed faintly detectable levels of NUB1L protein in only one of the clones (F9) and with only one of the antibodies (Fig. 3A and C). Densitometric evaluation of the NUB1L bands obtained by three dilutions of lysates on graded western blots (Fig. 3A) revealed that the residual expression of NUB1L protein in the shRNA transfectants was 2% (G6), 8.5% (F9), and 4% (H10), respectively.

Next, we transiently transfected the three NUB1L knock-down clones and HeLa-cells with an expression construct for HA-FAT10-DHFR and monitored its degradation in pulse-chase experiments. As shown in Fig. 4, the degradation of HA-FAT10-DHFR was markedly slowed down in the three NUB1L knock-down clones compared to HeLa-cells and the reduction in HA-FAT10-DHFR degradation correlated with the extent of NUB1L deficiency. This result strongly suggests that also in intact cells, NUB1L is required to support the degradation of FAT10-linked proteins by the proteasome.

Degradation by the proteasome can be divided into two categories: inducible destruction, which is chiefly mediated by attachment of a K48-linked polyubiquitin chain to target proteins, and degradation of proteins which contain intrinsic targeting signals. Proteins with loosely folded domains can be degraded by the 20S proteasome without the need for ubiquitylation or ATP-dependent unfolding. The structure of FAT10 remains yet to be solved, however, the high sequence similarity to ubiquitin and ISG15 suggests that it is composed of two ubiquitin-fold domains [17], which are anything but loosely folded. One can thus safely assume that FAT10 has evolved as a component of a specialized regulatory system responsible for targeting a subset of proteins for proteasomal degradation, and is not merely degraded due to an intrinsic instability. This notion is further supported by the finding that purified 20S proteasome was unable to degrade FAT10-DHFR in vitro (Fig. 2C), and that C-terminal attachment of FAT10 did not promote degradation (data not shown).

The important role for NUB1L in mediating the degradation of FAT10-linked proteins in vitro has been confirmed in three

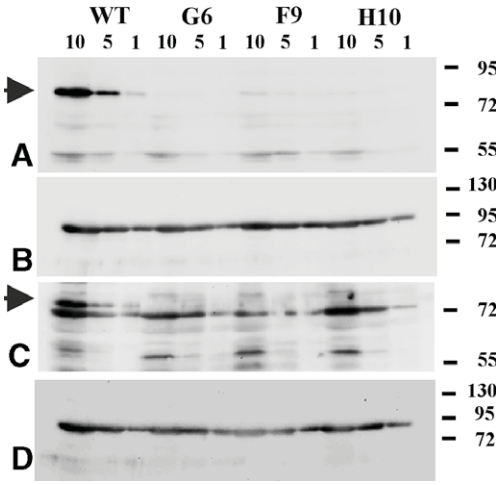

Fig. 3. Efficient knock down of NUB1L in stable HeLa transfectants expressing two NUB1L shRNAs. Shown are western blots of wild type HeLa-cells (WT) and three different stable transfectants expressing two NUB1L specific shRNAs (clones F9, G6 and H10). 10, 5, and 1 μl of cell lysate (indicated above the panels) were loaded for each cell type. (A and C) Western blots probed with two different NUB1/NUB1L peptide-specific antibodies (A, Ref. [13]; C, Biomol). The arrowheads show the position of NUB1L. (B and D) Loading control for panels A and C with anti-HSP 90 antibody, respectively.

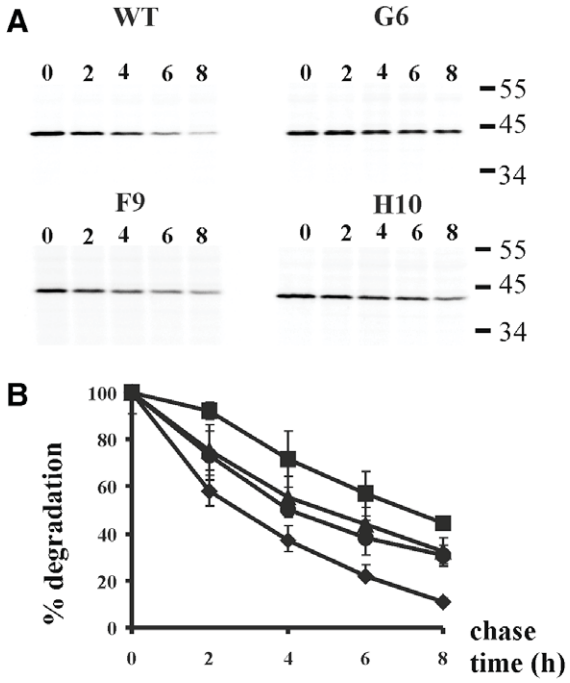

Fig. 4. Knock down of NUB1L attenuates the degradation of HA-FAT10-DHFR. (A) Shown are autoradiograms of HA-FAT10-DHFR immunoprecipitations from HeLa-cells (WT) and the NUB1L shRNA transfected clones G6, F9, und H10 described in Fig. 3. Cells were pulse labeled with [^{35}S]-Met/Cys for one hour and chased for the indicated time periods (in h). (B) Graphic representation of the phosphorimager-assisted quantitative evaluation of three different experiments as exemplified in panel A; the extent of FAT10 degradation in percent is plotted versus the chase time. Shown are the mean ± S.D. for HeLa wild type cells (diamonds), and the transfectants F9 (dots), H10 (triangles), and G6 (cubes).

independent HeLa clones stably expressing two different NUB1L shRNAs each. Compared to the parental cells, the degradation rate of HA-FAT10-DHFR was reduced by approximately 50% (Fig. 4) thus indicating that the expression of NUB1L mRNA and protein, which was reduced by over 90%, became a rate-limiting factor for HA-FAT10-DHFR degradation (Fig. 3). It hence appears, that both in vitro and in vivo, NUB1L is indispensable for normal degradation of FAT10-DHFR. We have shown previously that the N-terminal ubiquitin-like domain of NUB1L is required for the acceleration of FAT10 degradation and we speculate that binding of this domain to the 26S proteasome induces a conformational change in the 19S regulator which specifically facilitates degradation of FAT10-modified substrates while the degradation of poly-ubiquitylated proteins is not affected [11].

Combined with our studies on FAT10 degradation in ts20 cells and the fact that deletion of all lysines of FAT10 had no effect on FAT10 degradation [8], the present in vitro studies establish FAT10 as the second member of the ubiquitin family that serves as an autonomous signal for targeting proteins for degradation by the 26S proteasome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.01.006.

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